IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICANT	: Salter *et al.*
INVENTION	: **Method for Modification of NMDA Receptors Through Inhibition of Src**
SERIAL NUMBER	: 10/814,109
FILING DATE	: March 30, 2004
EXAMINER	: Standley, Steven H.
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DECLARATION UNDER 37 CFR § 1.132

I, Kenneth A. Pelkey, do hereby declare as follows:

1. I am a Post-Doctoral Research Fellow in the Laboratory of Cellular and Synaptic Neurophysiology at the National Institute of Child Health and Human Development (NICHD) in Bethesda, Maryland. Currently, my work includes investigating cell signaling mechanisms responsible for high frequency stimulation-induced LTD (long-term depression) of glutamate release at mossy fiber inputs onto interneurons within the CA3 region of the hippocampus. During the years 1997-2002, I was a graduate student in the laboratory of Dr. Michael Salter, an inventor in the above-referenced application entitled **"Method for Modification of NMDA Receptors Through**

Inhibition of Src", having U.S. Application Serial No. 10/814,109, filed March 30, 2004. As a graduate student I participated in numerous experiments examining the regulation of NMDA(N-methyl-D-aspartate) receptor function, including the experiments described in the abstract of the poster presentation entitled "ND2, a mitochondrially-encoded protein, interacts with Src Kinase at the NMDA receptor complex" (Gingrich et al. Society for Neuroscience Abstract, 2001).

2. The figure attached hereto shows a scaled-down replica of the poster described in the abstract.

3. In the Office Action mailed on September 11, 2006, claims 6, 9-10, 26 and 29 were rejected under 35 USC 102(b) as anticipated by, or in the alternative, under 35 USC 103(a) as obvious over Gingrich et al. (abstract of the poster presentation entitled "ND2, a mitochondrially-encoded protein, interacts with Src Kinase at the NMDA receptor complex" Society for Neuroscience Abstract, 2001).

4. Furthermore, in the Office Action mailed on September 11, 2006, claims 6-10, 26-29 and 32-35 were rejected under 35 USC 103(a) as obvious over the Gingrich abstract further in view of Schwarze et al. (Science 285:1569-1572 1999).

5. The Gingrich abstract discloses that the unique domain of tyrosine kinase Src binds to the ND2 protein to upregulate NMDA receptor function and further discloses that this binding is prevented by a peptide corresponding to amino acid residues 40-58 of the Src unique domain. However, Gingrich does not teach a specific binding region for this peptide. Furthermore, Gingrich

does not discuss any *in vivo* analgesic effect of the inhibition nor does Gingrich mention transduction of the peptide or TAT fusion techniques.

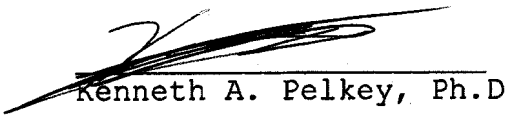
6. The instant invention, as currently claimed, is drawn to a composition containing a peptide, designated as SEQ ID NO:2, combined with a pharmaceutically acceptable solution. The residues of the peptide correspond to amino acid residues 40-49 of the Src unique domain and are fused to amino acid residues of the transduction domain of the human immunodeficiency virus (HIV-TAT). Once the composition is administered, the peptide is transported into the cellular interior by the TAT domain and binds to ND2 protein. This binding inhibits the interaction between Src and ND2 to downregulate the NMDA receptor.

7. Gingrich does not disclose that the peptide binds specifically at amino acid residues 40-49 of the Src protein. The instant inventors identified the specific binding sequence by examining the binding properties of different subpeptides derived from the Src40-58 amino acid sequence. The binding region, at amino acid residues 40-49 of the Src unique domain, was identified by data gathered from experimentation performed <u>after</u> the presentation of the cited poster/abstract.

8. Accordingly, one would not be able to discern the peptide of the claimed composition, SEQ ID NO:2, from the disclosure of Gingrich.

The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issuing thereon.

Feb 8 '07
Date


Kenneth A. Pelkey, Ph.D.

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ND2, A MITOCHONDRIALLY ENCODED PROTEIN, INTERACTS WITH SRC KINASE AT THE NMDA RECEPTOR COMPLEX.

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The Hospital for Sick Children

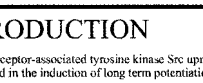
INTRODUCTION

The receptor-associated tyrosine kinase Src upregulates NMDA receptor function and is involved in the induction of long term potentiation (LTP) in the hippocampus.

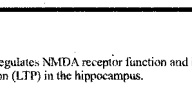
Structure of Src kinase:

Sequence alignment of AA40-58:

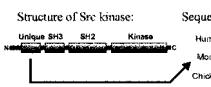
A. Src40-58 peptide prevents the Src mediated upregulation of NMDAR function.



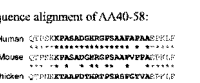
B. Src40-58 peptide prevents LTP induction.



C. Src40-58 peptide prevents the association of Src with NMDARs.



D. Proposed Model:



METHODS

Yeast two-hybrid screen

The yeast two-hybrid system was provided by R. Brent, Massachusetts General Hospital, Boston, MA (Gyuris *et al.*, Cell 75:791-803 (1993)). The an que domain of mouse n-Src was used to screen a human fetal brain cDNA poo fusion library, and cDNAs encoding potential Src unique domain interacting proteins were isolated and sequenced. Three clones encoded amino acids (aa) 100 to 347 of ND2, and two clones encoded aa 48 to 267.

Src, Fyn, and ND2 recombinant proteins

The unique domains of Src and Fyn in pGEX2T^6 (Amersham Pharmacia) were provided by M. Woodrow, Sugen Inc. (Redwood City, CA). The cDNAs encoding the SH3 and SH2 domains of mouse n-Src and Fyn were subcloned into pGEX4T-1 (Amersham Pharmacia). To create ND2.1, ND2.2, and ND2.3 GST fusion proteins, cDNAs encoding aa 239-321 (ND2.1), aa 189-238 (ND2.2), and aa 1-188 (ND2.3) of human ND2 were subcloned into pGEX4T-1. GST fusion proteins were purified by glutathione affinity chromatography from bacteria transformed with plasmid DNA.

In vitro binding assays

To detect binding of the Src unique domain with ND2 GST fusion proteins, recombinant Src unique domain was purified on glutathione Sepharose, removed by thrombin cleavage, biotinylated, and incubated with GST-ND2.1, GST-ND2.2, or GST-ND2.3 on beads. The beads were washed and the samples were resolved by SDS-PAGE, transferred, and probed with streptavidin-HRP (Sigma Chemical Co.). The same method was used to detect the binding of biotinylated domains of Src and Fyn with GST-ND2.1 on beads. In competition experiments, Src40-58 or scrambled Src40-58 peptide (final concentration 10 µg/ml) was pre-incubated with GST-ND2.1 on beads before addition of biotinylated Src or Fyn domains. Peptides were synthesized by J. Bell, Ottawa Regional Cancer Centre, Ottawa, Ontario.

Co-immunoprecipitation of ND2 and Src from various tissues

Total soluble protein was prepared from rat tissues by homogenization in sucrose/HEPES buffer, with NP-40 added to 1%. Samples were incubated with antibodies overnight at 4°C, and immune complexes were isolated by the addition of protein G-Sepharose, followed by incubation for 2 h at 4°C. The beads were washed and the samples were resolved by SDS-PAGE, transferred, and analyzed by immunoblotting. Anti-ND2 pAb was from Dr. R.F. Doolittle (UCSF, CA) and anti-Src mAb 327 was from J. Bolen (DNAX, Palo Alto, CA).

Cellular fractionation and immunoblotting and pull down assays

PSD proteins were prepared as described (Kennedy *et al.*, PNAS (USA) 80:7357-61, 1983). Cellular fractionation of rat brain tissue was performed by differential centrifugation of homogenate in sucrose/HEPES buffer; the light mitochondrial fraction was used in subsequent experiments. For immunoblots, samples were resolved by SDS-PAGE, transferred, and probed with anti-ND2, anti-Cytol mAb 1D6-E1-A8 (Molecular Probes), anti-ND4 mAb 20C11-B11-B11 (Molecular Probes), anti-NR1 mAb 54.1 (BD Transduction Labs), anti-Src, or anti-synaptophysin mAb SVP-38 (Sigma). For pull down assays, solubilized proteins from PSDs were incubated with GST-ND2.1, GST-ND2.2, GST-ND2.3, or GST alone on beads. The beads were washed and the samples resolved by SDS-PAGE, transferred, and probed with anti-Src or anti-GST mAb Z-5 (Santa Cruz Biotechnology Inc.).

Immunoprecipitations from PSD preparations

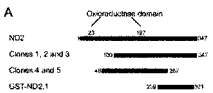
Solubilized proteins from PSDs were incubated with antibodies overnight at 4°C, and immune complexes were isolated by the addition of protein G-Sepharose followed by incubation for 2 h at 4°C. The beads were washed and the samples were resolved by SDS-PAGE, transferred, & probed with anti-ND2, anti-Src, anti-phosphotyrosine mAb 4G10 (Upstate Biotech), or anti-NR1. In competition experiments, Src40-58 or scrambled Src40-58 peptide (final concentration 30 µg/ml) was incubated with the starting material before adding the antibodies used for immunoprecipitation.

Electrophysiological recordings from hippocampal neurons treated with chloramphenicol

Whole cell electrophysiological recordings were made from cultured hippocampal neurons using standard methods. For control chloramphenicol treatment, media was removed from the culture dishes, the neurons were rinsed, and then bathed in extracellular solution supplemented with 50 µg/ml chloramphenicol to block mitochondrial translation. For neurons treated for 48 h, growth media was supplemented with 50 µg/ml chloramphenicol 48 h prior to electrophysiological recording.

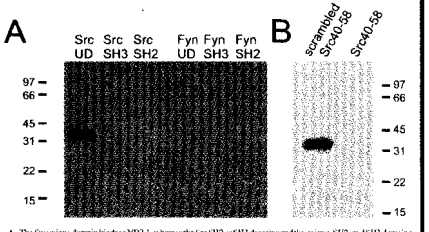
RESULTS

Figure 1. The Src unique domain binds to the C-terminus of ND2.



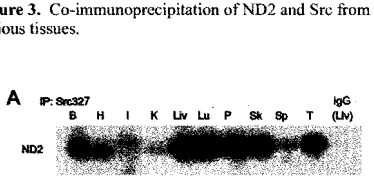
A. Schematic diagram showing the structure of ND2, clones isolated from the yeast two-hybrid screen, and recombinant GST-tagged ND2 fusion proteins. **B.** The Src unique domain binds to ND2.1, and not to ND2.2, ND2.3, or GST alone. The Src unique domain was purified as a GST fusion protein, purified after bacterial expression and cleaved from the GST moiety using thrombin. The Src unique domain was then biotinylated and incubated with GST-ND2.1, GST-ND2.2, GST-ND2.3, or GST alone bound to beads. The proteins were resolved by SDS-PAGE, transferred, and probed with streptavidin-HRP.

Figure 2. The Src unique domain binds to ND2.1, and this interaction is prevented by Src40-58 peptide.



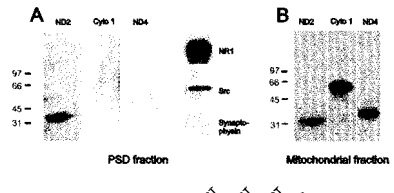
A. The Src unique domain binds to ND2.1, whereas the Src SH2 or SH3 domains and the unique, SH2, and SH3 domains of Fyn do not bind to ND2.1. **B.** The binding of the Src unique domain to ND2.1 is prevented by Src40-58 peptide. Src and Fyn domains were prepared as GST fusion proteins, purified after bacterial expression and cleaved from the GST moiety using thrombin. The proteins were biotinylated and then mixed with GST-ND2.1 on beads. The proteins were resolved by SDS-PAGE, transferred, and probed with streptavidin-HRP. In **B** the ND2.1 pull down of the Src unique domain was done in the presence of Src40-58 or scrambled Src40-58 peptide.

Figure 3. Co-immunoprecipitation of ND2 and Src from various tissues.



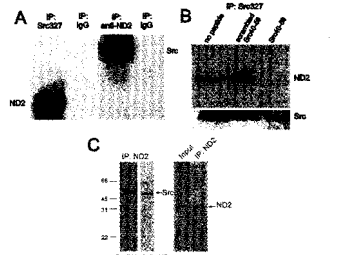
A. ND2 and Src co-immunoprecipitate from various rat tissues. Tissues: B - brain; H - heart; I - intestine; K - kidney; Liv - liver; Lu - lung; P - pancreas; Sk - skeletal muscle; Sp - spleen; T - testis. Total soluble protein samples were quantitated, and equal amounts of protein from the various tissues were incubated with anti-Src antibody. Immune complexes were isolated following incubation with protein G-Sepharose beads. The beads were washed, and the samples resolved by SDS-PAGE, transferred, and probed with anti-ND2 antibody.

Figure 4. ND2 is present in the PSD fraction obtained from rat brain.



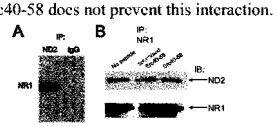
A. ND2, but not cytochrome oxidase I (Cyto 1) or ND4, is present in PSD proteins. PSD proteins were prepared from rat brain homogenate, resolved by SDS-PAGE, and immunoblotted for ND2, Cyto 1, or ND4. The PSD preparation contained NR1 and Src but the presynaptic marker synaptophysin was absent. **B.** Mitochondrial proteins were similarly prepared, resolved, and probed for ND2, Cyto 1, or ND4. Note that cytochrome oxidase I and ND4 are strongly detected in the mitochondrial fraction but not in the PSD fraction. **C.** The ND2.1 GST fusion protein pulls Src from PSD preparations. ND2.1 bound to beads was incubated with PSD proteins. The beads were washed, and bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Src and anti-GST antibodies.

Figure 5. ND2 and Src interact within post-synaptic densities, and this interaction is prevented by Src40-58



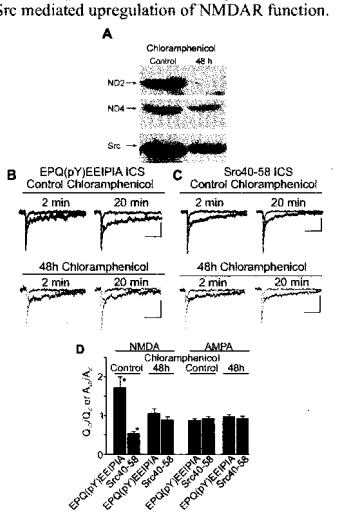
A. ND2 and Src co-immunoprecipitate from PSD preparations. **B.** Co-immunoprecipitation of ND2 and Src is prevented by Src40-58 peptide but not by scrambled Src40-58. Antibodies used for immunoprecipitation and immunoblots are indicated. **C.** ND2 is not a phosphorylation target of Src. Anti-ND2 co-immunoprecipitation samples from PSD preparations were immunoblotted for tyrosine phosphorylated proteins (4G10), Src, or ND2. Note that the only tyrosine phosphorylated protein has a molecular weight that corresponds to that of Src. ND2 itself is not tyrosine phosphorylated.

Figure 6. ND2 also co-immunoprecipitates with NMDAR subunits, but Src40-58 does not prevent this interaction.



A. ND2 co-immunoprecipitates with NMDA receptor subunits. **B.** The co-immunoprecipitation of ND2 and NMDA receptor subunits is not prevented by Src40-58 peptide.

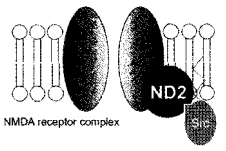
Figure 7. Chloramphenicol inhibits ND2 production and prevents Src mediated upregulation of NMDAR function.



A. Inhibition of ND2 production in cultured rat hippocampal neurons treated with chloramphenicol for 48 h. Immunoblots were probed as indicated. **B.** Chloramphenicol prevents the upregulation of NMDAR function, as measured by mEPSCs recorded from neurons in the presence of Src activator peptide. **C.** Chloramphenicol also prevents Src40-58 peptide from reducing the NMDAR component of mEPSCs. **D.** Summary of electrophysiology data. NMDA component data was calculated as Q_{-70}/Q_{+40}, whereas AMPA component data was calculated as A_{-70}/A_{+40}.

CONCLUSIONS

- Our data indicate that ND2 is localized at post-synaptic densities where it acts as an adapter protein to recruit Src to the NMDAR complex to modulate NMDA channel function. The interaction of ND2 and Src is unexpectedly mediated through the unique domain of Src, a region of the protein that may have unsuspected importance in protein-protein interactions.
- Revised model: ND2 as an adapter protein.



- The interaction of ND2 with the NMDAR complex and the export of ND2 to synaptic sites is under investigation.
- The interaction of Src and ND2 has implications for a broad range of cellular activities including proliferation, differentiation, cell-cell contact, and maintenance of the cytoskeleton in neurons and other cell types.

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CIHR IRSC